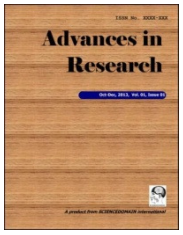

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Multiple Antibacterial Activities of Proteinaceous Compounds in Crude Extract from the Eastern Subterranean Termite, *Reticulitermes flavipes* Kollar (Blattodea: Isoptera: Rhinotermitidae)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SJS, XPH and XQY designed the study, wrote the protocol. Author YZ wrote the first draft of the manuscript, conducted the experiments and data analysis. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To assess the presence of antibacterial activities in *Reticulitermes flavipes* against a common Gram-positive soil bacterium *Bacillus subtilis*; to determine the nature of antibacterial compounds of crude extracts; and to analyze the size profile of active compounds.
Place and Duration of Study: Department of Entomology and Plant Pathology and Department of Biological Sciences in Auburn University between August 2012 and July 2013.
Methodology: The cell free crude extracts were obtained from *R. flavipes* workers. Five size-fractionated solutions (≥100, 30-100, 10-30, 3-10, and ≤3 kDa) were achieved by sequentially size fractionating the crude extract with Microsep™ Advance Centrifugal

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Devices. Heat-stable fraction was acquired by subjecting the crude extract to heat-treatment to denature proteins. Activities of the crude extract, heat-treated extract, and size-fractionated extracts against *B. subtilis* were determined using the inhibition zone assay. Anti-bacterial activities were determined by the measurements of the diameters (mm) of growth inhibition zones. The significances among the seven treatments, in comparison to Ampicillin (positive control) and Tris-NaCl buffer (negative control), were determined using repeated measures ANOVA.

Results: The activity against *B. subtilis* was evidenced in all but the heat-treated solutions, indicating the presence of antibacterial activities, the existence of multiple active compounds in the crude extracts, and the protein nature of the active compounds. The active compounds, with the molecular sizes ranging from <3 to >100 kDa, demonstrated different levels of antibacterial activity ($P = 0.017$). The greatest activity was observed in the fraction of 3-10 kDa and Ampicillin, followed by the fractions of ≤3 kDa and ≥100 kDa, and the lowest in the fraction of 10-30 kDa.

Conclusion: Crude extracts from *R. flavipes* workers contain multiple proteins with various antibacterial activities against a common Gram-positive soil bacterium *B. subtilis*.

Keywords: Termite immunity; antibacterial activity; Reticulitermes flavipes; Bacillus subtilis; proteins; peptide.

1. INTRODUCTION

With roughly two million species, insects account for one of the most successful evolution groups [1]. They colonize nearly all ecological niches and feed on most of plants and animals. Consequently, insects have evolved effective innate immune systems in confronting a large variety of potentially harmful microorganisms. Their innate immune systems may comprise of a series of cellular and humoral reactions, which differ from the adaptive immune system of vertebrates [2].

The innate immune system of termites has been of great interest for discovering novel compounds against microbes, as well as exploring new approaches to control termites. Subterranean termites (Blattodea: Isoptera: Rhinotermitidae), especially species of *Reticulitermes* genus, have a wide distribution in the U.S. (except Alaska). They nest and forage underground in soil environments rich in pathogenic microbial communities [3,4]. Interacting with many soil pathogens has led to the development of disease resistance mechanisms that allowed termites to survive and to develop in such environment.

Several antimicrobial proteins/peptides have been isolated or identified from subterranean termite salivary glands and hemolymph [5,6,7,8]. Termicin, β-1, 3-glucanase and termite Gram-negative binding proteins (tGNBPs) are reported as antifungal compounds in several *Reticulitermes* species, and lysozyme as antibacterial compound in *R. speratus* [5,6,7,8]. However, there has been no report on antibacterial activity from the eastern subterranean termite, *R. flavipes* Kollar *(*Isoptera: Rhinotermitidae), the most common economically important wood destroying pest in the southeastern United States.

This study has a three-fold objective: 1) to assess the presence of antibacterial activities in *R. flavipes* against a common Gram-positive soil bacterium *Bacillus subtilis*; 2) to determine the nature of antibacterial compounds of crude extracts; and 3) to analyze the size profile of active compounds. The ultimate goal is to discover new antibacterial compounds for development of antibiotic drugs for treating antibiotic-resistant infections.

2. MATERIALS AND METHODS

2.1 Organisms

R. flavipes was collected on the Auburn University campus (Alabama, USA) between August 2012 and March 2013. Termite collections were maintained in Urban Entomology Laboratory at 25ºC for at least 20 days before subjected to crude extraction.

Gram-positive bacterium *B. subtilis* (ATCC 6633) was obtained from Microbiology Teaching Laboratory of Auburn University and stored in skim milk at -80℃.

2.2 Whole Body Extraction and Size Fractionating

For each extraction, termite workers (5 g) were suspended in 25 ml of 20 mM Tris-HCl, 20 mM NaCl (pH = 7.5) buffer and homogenized (Sonic Dismembrator Model 100, Fisher Scientific, Pittsburgh, PA) on ice for 30 sec. The lysed extract was centrifuged twice at 8,000 *g* (Beckman JA-21, Beckman Coulter, Inc. Brea, CA) and 4ºC, each for 20 min, to remove insoluble materials.

The resulting cell free extract (15 ml) was sequentially size fractionated with Microsep^{TM} Advance Centrifugal Devices (Pall Corporation, Port Washington, NY) to obtain five fractions (≥100, 30-100, 10-30, 3-10, and ≤3 kDa). Protein concentrations of the crude extracts and size-fractionated solutions were determined by Bradford assay [9] with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The fractionated solutions were lyophilized (Heto Lyolab 3000, Thermo Scientific, Waltham, MA) at -57ºC overnight and dissolved in Milli-Q water to achieve the final protein concentration of approximately 5 mg/ml, as same as the crude extract.

2.3 Heating Treatment

To determine the nature of the active antibacterial compounds, a sample (5 ml) of the crude extract was subjected to heat treatment at 100ºC for 10 min. The resulting solution was centrifuged at 8,000 *g* (Beckman JA-21, Beckman Coulter, Inc. Brea, CA) and 4ºC for 10 min to remove denatured proteins.

2.4 Inhibition Zone Assay

Activity of the crude extract, heated crude extract (supernatants), and size-fractionated solutions against *B. subtilis* was determined using a modified inhibition zone assay, also named Kirby-Bauer Disk Diffusion method [10,11]. In brief, approximately 2 x 10^8 *B. subtilis* cells grown to log-phase (OD_{600} of 0.3) were mixed with 2.5 ml of soft agar and overlaid on a Lysogeny Broth (LB) agar plate. Three filter paper disks (5 x 5 mm) were placed uniformly on the bacterial lawn in each plate. The paper disks were treated with one of the following samples, respectively: 20 µl of the seven termite extract treatments (crude, heat-treated, and the five size-fractions); 1 µl of ampicillin (25 mg/ml) as positive control, or 20 µl of 100 mM Tris-HCl, 100 mM NaCl (pH = 7.5) buffer as a negative control. All plates were incubated at 37℃ for 24 h to allow bacterial growth.

The experiment was repeated three times, each with 3 replicates (N = 9).

2.5 Statistical Analysis

The diameters (D; mm) of growth inhibition zones were measured and compared using repeated measures ANOVA (PROC GLM; α = 0.05; SAS 9.2) to determine the significance among treatments.

3. RESULTS AND DISCUSSION

The results are presented in Table 1. The clear inhibition zone in the crude extract treatment shows the presence of activity against *B. subtilis* in *R. flavipes*.

Table 1. Diameter (mm) of clear inhibition zone (N = 9) on *B. subtilis* soft agar plate after 24 h incubation at 37ºC

Treatments	Diameters of inhibition zone* (Mean ±SD)
≥100 kDa	14.68±0.78[b]
30-100 kDa	11.96±0.54[c]
10-30 kDa	8.25±0.17[d]
3-10 kDa	20.58±0.53[a]
≤3 kDa	16.32±0.83[b]
Crude extract	13.76±0.80[b]
Heated crude extract	0[e]
100 mM Tris-HCl, 100 mM NaCl buffer	0[e]
Ampicillin	21±1.83[a]

**Different letters in the column indicate significant differences among the samples*

The absence of clear inhibition zone of the heat-treated crude extract indicates the proteinaceous nature of the active compounds in crude extract. However, this absence of activity cannot be used as a conclusive evidence to exclude the possibility of non-proteinaceous active molecules in the crude extract, because it is possible that the proteinaceous active molecules in the samples are too low in concentrations to show their activity in the inhibition zone assays. Future work is needed to elucidate this possibility.

An interesting finding of this study is that there are multiple compounds in the crude extract possessing potent antibacterial property, as evidenced by the clear inhibition zones in all the five size-fractions. The molecular sizes of the active compounds range from <3 to >100 kDa. The different measurements of clear inhibition zones in the five size-fractions show that the level of antibacterial activity varies with the molecular size of the protein/peptide. The greatest antibacterial activity is displayed in the fraction of size 3-10 kDa, which has a comparable activity as Ampicillin, and the lowest activity in the fraction of size 10-30 kDa (F=26.4, P=0.016). This study is the first to report the antibacterial activities of multiple compounds existing simultaneously in a subterranean termite species.

Another interesting finding of this study is the antibacterial activity of compounds in the ≤3 kDa fraction. Of the known antimicrobial proteins/peptides in subterranean termite, most have antifungal activities [6,7,8,11]. The only protein reported having antibacterial activity is lysozyme identified from a different termite species (*R. speratus*) [6]. However, lysozyme has a molecular size of 14.5kDa, bigger than 3 kDa. The only documented antibacterial compound smaller than 3 kDa is spinigerin (~2.5-3 kDa). Spingerin is a broad-spectrum antibacterial peptide reported from a tropical and subtropical fungus-growing termite,

Pseudacanthotermes spiniger (Isoptera: Macrotermitinae) [5]. It is possible that spinigerin is present in *R. flavipes* because it is reported to be highly conserved. However, spinigerin is reported inactive against *B. subtilis* [5].

Therefore, it is highly likely that the multiple antibacterial proteins/peptides, including the small peptides of represent compounds that haven't been identified, and the small peptides ≤3 kDa, reported in this study are molecules that have not been reported and identified.

Up to date, no study has directly determined the mode of action (MOA) of the two termite-derived antimicrobial peptides, spinigerin and termicin [5]. Because the α-helical structure spinigerin has a strong electrostatic attraction between its three Arg residues and the negatively charged polar head groups of the phospholipids on the bacterial membrane surface, Lee et al. [12] suggested a MOA of spinigerin breaking down membrane and consequent cell death. Da Silva et al. [13] proposed that the antifungal properties of termicin may be related to its marked hydrophobicity and its amphipathic structure as compared to other antibacterial defensins.

In this study, unsterilized termite bodies were used to obtain crude extract. The antibacterial activities may come from proteins from associated bacteria on termite cuticle, symbiotic protists in termite gut, or the termite itself (regardless of whether it is the hemolymph, specific organs or glands). Formosan subterranean termite, *Coptotermes formosanus*, has cuticle bacteria (*Pseudomonas aeruginosa*, *Serratia marcescens*, *Cedecea davisae*, and *Lysinibacillus sphaericus*) showing antifungal activities or antibacterial effect on an entomopathogenic pathogen *B. thuringienisis* [14], and a bacterium (*Streptomyces* sp) associated with fecal nest protects termite against entomopathogens [15]. Several protists isolated from the guts of several termite species (*Macrotermes michaelseni, C. formosanus,* and *R. speratus*) are reported producing antibiotics against bacteria including *Bacillus* spp., *Escherichia coli* and *Staphylococcus aureus* [16,17,18]. Future work will identify the source or the origin of the antibacterial compounds.

4. CONCLUSION

In conclusion, crude extracts prepared from the Eastern Subterranean termite *R. flavipes* contain multiple proteins/peptides with antibacterial activity against a common Gram-positive soil bacterium *B. subtilis*.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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